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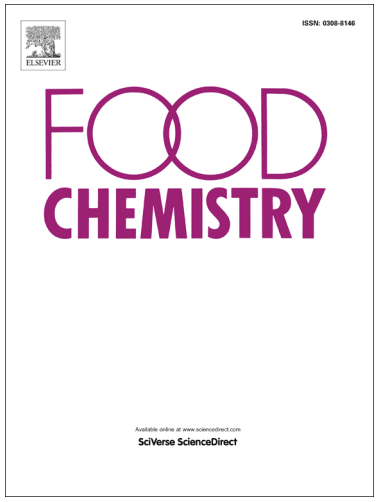

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Impact of rapeseed press-cake on Maillard reaction in a cookie model system

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Abstract

Rapeseed press-cake (RPC) is a byproduct of rapeseed oil production, rich in proteins and fiber. The aim of this study was to investigate the impact of cold pressed RPC, RPC fiber isolate and RPC alkaline extract on the formation of acrylamide and 5-hydroxymethylfufural (HMF) in cookies. Both compounds were influenced by the ingredients: the addition of RPC led to a significant dose-dependent increase of HMF in the cookies and to an increase of acrylamide up to 66.9%. On the contrary, acrylamide concentration was reduced down to 39.6% in presence of the alkaline extract and down to 4.4% in the presence of the fiber extract. The Michael addition of free amino acids to acrylamide was further investigated by high-resolution mass spectrometry (HRMS) revealing that cysteine was the preferred nucleophile for acrylamide elimination.

Keywords: acrylamide, 5-hydroxymethylfurfural, Maillard reaction, rapeseed press-cake, Michael addition.

Abbreviations used: rapeseed press cake (RPC); Maillard reaction (MR); 5-hydroxymethylfurfural (HMF); high resolution mass spectrometry (HRMS); liquid chromatography (LC); water holding capacity (WHC); total phenolic content (TPC); bovine serum albumin (BSA).

List of compounds: acrylamide (PubChem CID: 6579), acrylamide-*d3* (PubChem CID: 12209671), 5-hydroxymethylfurfural (PubChem CID: 237332).

1. Introduction

The worldwide production of rapeseed increased by a factor of 2.4 over the last 20 years thus making rapeseed the most cultivated oilseed crop in Europe with a production of 19.5 M tons in 2012. The increase in rapeseed production is due to two major aspects: the expansion of biodiesel production from rapeseed and the improvement of the nutritional composition (e.g. decrease of erucic acid levels and glucosinolate levels) of rapeseed seeds (EFSA, 2013). Rapeseed press-cake (RPC) is a byproduct of rapeseed oil production and is mainly used in animal feed. However, RPC contains notable amounts of phenolic acids and tannins, as reviewed by Naczk and coworkers (1998); cold-pressed RPC is rich in crude fiber (around 11.2%) and protein (approximately 28.0 %) hence providing high potential to be used in human diet (Leming & Lember, 2005). Indeed, a novel rapeseed protein isolate extracted from canola varieties (*Brassicaceae napus L.* and *Brassicaceae rapa L.*) was adjudged to be safe and suitable for human diet by the EFSA (EFSA, 2013). Palermo and co-workers (2012) showed that the addition of a similar product based on soy proteins (okara) to a cookie model system was positively correlated to higher concentrations of acrylamide (+60%), *N*ε-(carboxymethyl)-L-lysine (+400%), and HMF (+100%). These compounds are commonly used as markers of the Maillard reaction (MR) that along with lipid oxidation, sugar caramelization and ascorbic acid oxidation, occupies a prominent place in nonenzymic browning. Beside the formation and degradation of the Amadori compounds, during the intermediate and advanced stage of the reaction, cyclization, dehydration, retroaldolization, rearrangement, isomerization, and further condensation take place. In the final/advanced stage, melanoidins and brown pigments are formed (Hellwig & Henle, 2014).

The Maillard cascade leads to hundreds of molecules, some of these compounds are useful since they are responsible for color and flavor formation or they participate to texture. Some other Maillard reaction end products (MRPs) can contribute to the off-taste of foods or have potential

negative effects on human health; an example is the formation of acrylamide and HMF which are two of the most-studied MRPs (Capuano & Fogliano, 2011).

Acrylamide is formed upon the reaction of asparagine with reducing sugars or from cleavage products (i.e., 2-butanedione, 2-oxopropanal) at temperature higher than 100 °C in low moisture conditions or after prolonged thermal treatment (Mottram, Wedzicha & Dodson, 2002). Cysteine and methionine in presence of reducing carbonyls and acrolein in presence of ammonia are other precursors of acrylamide, even if the yield is lower than the asparagine/reducing sugars system (Stadler et al., 2002). Focusing on the asparagine/glucose system, there are several routes that lead to acrylamide formation. The first mechanism involves 3-aminopropionamide as reaction intermediate upon the formation of the Schiff base and the subsequent decarboxylation and hydrolysis via Strecker degradation (Granvogl, Jezussek, Schieberle & Koehler, 2004). The second mechanism includes the direct decomposition of the Schiff base via intramolecular cyclization forming the azomethine ylide that directly decomposes on cleavage of the C-N bond to give acrylamide and 1-amino-2-hexulose (Yaylayan, Wnorowski & Perez Locas, 2003). Due to the presence of an acryloyl group, acrylamide is highly reactive and it can polymerize or form Michael adducts with free amino group, thiols or other nucleophiles (Adams, Hamdani, Lancker, Méjri & De Kimpe, 2010; Koutsidis et al., 2009). Acrylamide has been classified as a Group 2A carcinogen by the International Agency for Research on Cancer (IARC) and a Category 2 carcinogen and Category 2 mutagen by the European Union; moreover the EFSA mentions acrylamide formation in foods as one of the major concerns (Friedman, 2015).

HMF is used as an indicator for the heating of carbohydrate containing foods (Ramírez-Jiménez, Guerra-Hernández & García-Villanova, 2000). Several mechanisms concur to its formation since it can arise from both caramelization and MR (Hollnagel & Kroh, 1998). HMF is formed as an intermediate from 1,2-enolization and dehydration of glucose or fructose under acidic conditions. The further dehydration and cyclization of 3-deoxyosone is the key step for the formation of HMF.

In acidic conditions, HMF is mainly formed at high temperature under dry and pyrolytic conditions; it can arise from fructose and sucrose via the formation of highly reactive fructofuranosyl cations (Perez Locas & Yaylayan, 2008). Beside the increase of temperature, there are other factors that influence the formation of HMF: the pH, the water activity (Oliviero, Capuano, Cämmerer & Fogliano, 2009) and the dehydration promoted by bivalent cations (Gökmen & Şenyuva, 2007). HMF shows mutagenic, hepatotoxic and carcinogenic effects *in vitro* even though a toxic effect in humans has not been confirmed, as yet. A daily exposure of 2 to 30 mg of HMF per person through a regular diet has been estimated, suggesting its reduction in foods as a relevant issue (Capuano & Fogliano, 2011).

In this paper, the effects of three different ingredients obtained from RPC were investigated in order to improve the quality of cookies by controlling the formation of acrylamide and HMF through the use of a food byproduct. In this respect, the formation of acrylamide adducts and its elimination via Michael reaction was evaluated by liquid chromatography high-resolution mass spectrometry (LC-HRMS) to tentatively identify the mechanisms beside acrylamide elimination.

2. Materials and methods

2.1 Chemicals

Cold pressed native rapeseed cake pellets from *Brassica napus* L. var. *catana* were provided by Summer Harvest® oil manufacturer Perthshire (UK), and were stored in an airtight closed plastic bag at room temperature. Wheat flour and sugar in customary quality were obtained from local stores. Palm oil was purchased from the Kerfoot Groop, (Yorkshire, UK). All the other baking ingredients were of analytical grade and purchased from Sigma-Aldrich (Saint Louis, MO). All chemicals used in this study were purchased from Sigma-Aldrich (Saint Louis, MO) and were of analytical grade, unless mentioned otherwise.

2.2 Ingredients extraction

2.2.1 Alkaline extraction

Alkaline soluble protein extraction was performed following a procedure previously described by Ghodsvali *et al.*, (2005) with some modification. Briefly, freshly ground native RPC powder was weighed into a beaker and distilled water was added at a w/v ration 1:20. The pH was adjusted to 11 with NaOH (0.1 M) and the suspension was stirred for 30 minutes at room temperature. After centrifugation (25 minutes, 0 °C, 2058 g) the supernatant was collected and adjusted to pH 6.5 with HCl (0.1 M). The precipitated pellet was separated by centrifugation (25 minutes, 0 °C, 2058 g) and the supernatant was collected. A second precipitation was conducted by adjusting the pH to 4.5. The pellets of both precipitation steps were merged and washed two times at a w/v ratio 1:3 with distilled water and freeze-dried.

2.2.2 Fiber extraction

The dietary fiber was obtained from the pellet which remained after the first extraction step of the alkaline soluble protein extraction. The pellets were washed 6 times with distilled water at ratio of 1:2.5 w/v and freeze-dried.

2.2.3 Water Holding Capacity (WHC) determination.

In order to determine the WHC, 1 g of the fiber was weighed into a centrifuge tube and mixed vigorously with 50 mL distilled water for 1 minute. The mixture was centrifuged for 40 minutes (2058 g at 20 °C). The supernatant was discarded and the centrifuge tube was kept inverted for 10 minutes. The supernatant was weighed and the results were expressed as grams of water per gram of fiber (Chen, Rubenthaler, Leung & Baranowski, 1988).

2.3 Preparation of the cookies

The cookies were prepared according to a recipe described in an American Association of Cereal Chemists (AACC) method 1054. The effect of the components from rapeseed press-cake was

evaluated by replacing 6.5 % and 12.9 % of the flour content with the alkaline extract, 1.3 %, 2.6 %, and 5.2 % of the flour content with the fiber extract, and 6.5 % and 12.9 % of the flour content with the native RPC, respectively (**Table 1**). Each dough was rolled between two bars with a height of 3 mm and were shaped in a disk of 30 mm diameter. To ensure repeatability of the experiment, the same amount of dough (17 g) was placed on the middle shelf of a laboratory oven (Memmert UM200, Schwabach, Germany) and in the center of the shelf and baked at 180 °C for 18 minutes.

2.4 Spectrophotometric assays

A methanol extract (60 % methanol) of the ground RPC was prepared at a w/v ratio of 1/10 (v/v) to determine the antioxidant capacity and the total phenolic content (TPC) of the rapeseed press-cake. In order to determine the content of soluble protein using Bradford assay the extracts were prepared the same way replacing the methanol with distilled water. The suspensions were subsequently centrifuged (2058 g, 10 minutes, 4 °C), and supernatants were diluted if necessary.

2.4.1 Determination of antioxidant capacity

Antioxidant capacity was determined using 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) reagent (ABTS assay). Trolox was used as a standard to prepare calibration solutions in a concentration range between 0.021 mg trolox eq/mL to 0.064 mg trolox eq./mL. Sample extracts and the calibration solutions were diluted with 7 mM ABTS solution in a ratio 1:10 (v/v). Absorbance was measured after 2.30 minutes at a wavelength of 734 nm using an UV/VIS-spectrophotometer (Shimadzu UV-1650 PC). The inhibition was calculated following the procedure described by Pellegrini *et al.* (2003).

2.4.2 Total phenolic content (TPC)

TPC was measured using the colorimetric Folin-Ciocalteu method, following the procedure of Singleton *et al.* (1999). Gallic acid was used as a standard to prepare calibration solutions in a concentration range between 0.043 mg gallic acid eq./mL and 0.130 mg gallic acid eq./mL. Sample

solutions were prepared as described before and mixed with 500 µL distilled water in a ratio 1:4 and 125 µL Folin-Ciocalteu solution was added. The mixture was vortexed and left for 6 minutes at room temperature. After 1.25 mL of 0.70 M aqueous sodium carbonate was added, the mixtures vortexed and left for 90 minutes at room temperature. The solutions were measured at 760 nm using an UV/VIS-spectrophotometer (Shimadzu UV-1650 PC).

2.4.3 Soluble proteins

The protein quantification was examined by Bradford assay following a method described by Ernst & Zor (2010) with some modifications. Standard solutions (BSA) and samples (RPC alkaline, RPC fiber and RPC), respectively, were mixed with 1 mL Bradford reagent (1:1) in an UV semi-micro cuvette (Fischerbrand, Thermo Scientific, Bremen, Germany) and the absorbance was measured at 595 nm wavelength using an UV/VIS-spectrophotometer (Shimadzu UV-1650 PC, Kyoto, Japan) after 15 minutes at room temperature.

2.5 Determination of glucose

Samples were dissolved in water and filtrated (0.45 µm, PTFE syringe filter, Fischerbrand) and analyzed by HPLC (SP8800 HPLC, Spectra-Physics) equipped with an autosampler (Spectra series AS100, Spectra-Physics) and were detected with a RI detector (SP6040 XR, Spectra-Physics). The mobile phase was 0.0025 M sulphuric acid at a flow rate of 0.6 mL/min under isocratic conditions and the stationary phase was an ion-exclusion column (300 mm × 7.8 mm, 8 µm, REZEX RPM-monosaccharide Pb^{+2} 8%, Phenomenex, Torrance, CA). Glucose was quantified using an external calibration in a concentration range between 0.1 g/L and 10 g/L.

2.6 Quantitation of HMF

HMF was determined following the procedure previously described (Fiore et al., 2012). Briefly, 0.5 g of the ground, freeze-dried cookies was weighed into a 50 mL centrifuge tube and 8.5 mL of 0.1

% formic acid in water was added. The suspensions were mixed vigorously and 500 µL Carrez A and 500 µL Carrez B were added. Then the samples were mixed thoroughly and centrifuged (2058 g for 10 minutes at 4 °C). The supernatant was collected; 5 mL 0.1 % formic acid was added to the pellets and further centrifuged (2058 g 10 minutes, 4 °C). This process was repeated once more to three centrifuge steps and the supernatants were collected. Before LC separation, the samples were filtered by using a 0.45 µm cellulose filter (Phenomenex, Torrance, CA), finally 20 µL was injected twice onto a polar endcapped C18 column (Synergi Hydro 4 µm, 250 x 4.60 mm, Phenomenex, Torrance, CA). HMF was detected at 280 nm by using HPLC-UV (P680 HPLC pump and DDA-100 diode array detector; Dionex, San Jose, CA) equipped with an autosampler (ASI-100, Dionex). The mobile phase A was water and mobile phase B was methanol; the following binary gradient (min)/(%B) (0/10), (14/70), (19/10), (25/10) was used with a flow rate of 0.8 mL/min. HMF was quantified using a calibration curve in a concentration range between 200 ppb to 5000 ppb (R^2 > 0.9997 according to the intraday and interday assays). Carryover, matrix effects and recovery were tested according to the procedure previously reported (Fiore *et al.*, 2012).

2.7 Quantitation of acrylamide

Acrylamide was quantified following the procedure previously reported (Troise, Fiore & Fogliano, 2014). Briefly, 100 mg of the freeze-dried, ground cookies was weighed into a 15 mL centrifuge tube and 100 µL Carrez A and 100 µL Carrez B as well as 125 µL of [2,3,3-*d3*]-acrylamide (20 ppm final concentration) and 4.7 mL distilled water was added to the samples. The mixtures were centrifuged (2700 g, 10 minutes, 4 °C) and accurately filtrated with 0.2 µm nylon filter (Phenomenex, Torrance, CA). Finally, 10 µL of each sample was injected on an Accela 1250 quaternary pump (Thermo Fisher, Bremen, Germany). The U-HPLC system was equipped with a thermostated (30°C) polar endcapped C18 column (Synergi Hydro, 150 × 2.0 mm, 4.0 µm, Phenomenex, Torrance, CA) and the following binary gradient of 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in methanol (mobile phase B) (min)/ (% B) (0/5), (2/5),

(4/80), (5/80) was used. The flow rate was 0.3 mL/min and the LC system was directly interfaced to an Exactive Orbitrap HRMS (Thermo Fisher Scientific) equipped with a heated electrospray interface (H-ESI II) operating in the positive mode and scanning the ions in the *m/z* range of 50–400. The resolving power was set to 50000 full with half-maximum (FWHM, *m/z* 200), resulting in a scan time of 1s. The automatic gain control was used to fill the C-trap and improve accuracy in mass measurements (ultimate mass accuracy mode, 5×10^5 ions); maximum injection time was 50 ms. The interface parameters were as follows: spray voltage, 4.5 kV; capillary voltage, 42.5 V; skimmer voltage, 14 V; capillary temperature, 300 °C; heater temperature, 250 °C; sheath gas flow, 40 (arbitrary units); and auxiliary gas flow, 6 (arbitrary units). Before the acrylamide analysis, the instrument was externally calibrated by infusion with a calibration solution that consisted of caffeine, Met-Arg-Phe-Ala (MRFA), Ultramark 1621, acetic acid and [2,3,3-*d3*]-acrylamide in a mixture of methanol/acetonitrile/water (2:1:1, v/v/v). Acrylamide was quantified using the internal standard technique by monitoring the ratio acrylamide/[2,3,3-*d3*]-acrylamide. The analytical performances were evaluated according to the procedure previously described (Troise, Fiore & Fogliano, 2014).

2.7.1 Formation of acrylamide adducts via Michael addition

Michael addition products of acrylamide and thiol or amino group such as cysteine, lysine, tryptophan, glycine and proline were investigated by using an in-house database developed according to the pathways of acrylamide elimination. Following the analytical procedure for acrylamide quantitation and the same LC-HRMS setup, specific molecular formulas and their respective *m/z* ratios were included in Exact Finder (Thermo Fisher Scientific, Bremen, Germany). The following parameters were selected: isotopic pattern and retention time for the identification signal to noise ratio higher than 5, mass tolerance set to 5 ppm. The procedure was used for all the aqueous extracts of cookie samples and all the signals below the threshold of 10^4 area counts were excluded.

2.8 Statistical analysis

Data were analysed by ANOVA using XLStat (version 2014.5.03, Addinsoft, NY). All the results were expressed as mean ± SD. Significant differences between the samples with a confidence interval of 95% were performed by using Tukey test. The average was calculated using the results of the thermal treatment replicates and the technical replicates (four observations per sample).

3. Results and discussion

3.1 Characterization of the press-cake and the components extracted from RPC.

The press-cake and its extracts were analyzed regarding their antioxidant capacity, HMF content, soluble protein content, and TPC content and the results were reported in Supplementary **Table 1.** Several studies indicate that the content of lysine, cysteine, and methionine of RPC as well as asparagine, and lysine of a protein isolate extracted using similar alkaline precipitation methods are higher than those of wheat flour (Von Der Haar, Müller, Bader-Mittermaier & Eisner, 2014).

The glucose levels of native RPC were in line with the glucose levels found in rapeseed meal (Fang, Peng, Tang, Liu, Dai & Jin, 2007) and were higher than the content of reducing carbohydrates contained in wheat flour (1.7 g/100 g), as reviewed by Palermo and co-workers (Palermo, Fiore & Fogliano, 2012). Glucose was not detected in the other two ingredients, alkaline extract and fiber extract.

In the native RPC, the TPC was two times lower than the levels of mg gallic acid eq/100 g (1.44 ± 0.22) found for rapeseed seeds (Terpinc, Čeh, Ulrih & Abramovič, 2012). The results on TPC were higher in the fiber extract than the alkaline extract, while opposite results were observed for the antioxidant activity. Traces of HMF were found in the alkaline extract (0.002 ± 0.000 mg/g) and the fiber extracts (0.004 ± 0.003 mg/g) probably due to the high pH used in the precipitation procedure. The WHC of the fiber extract from RPC (8.06 ± 0.23 g water/ g fiber) was in the same order of magnitude to the WHC of cellulose while it is notably higher than the WHC of cortical pea fiber

(5.66 g water/ g fiber) and chitosan (3.05 g water/ g fiber) (Palermo, Fiore & Fogliano, 2012). The carbohydrate fraction of rapeseed meal contains 26.8-28.4 % lignin, 12.6-12.9 % hemicelluloses, and 22.9-22.5% cellulose, besides others (Lomascolo, Uzan-Boukhris, Sigoillot & Fine, 2012). These polysaccharides can act as precursors of MRPs or they potentially bind water during the baking process and therefore reduces the water activity (Hollnagel & Kroh, 1998).

3.2 HMF and acrylamide in the model cookies

The HMF and acrylamide levels of the model cookies in our study are in line with the results reported in other quoted papers (Capuano & Fogliano, 2011; Fiore et al., 2012; Palermo, Fiore & Fogliano, 2012).

Independently of the ingredients used, no reduction of HMF was observed with the different recipes toward the control cookies and, in this respect, different mechanisms were hypothesized to concur to the increase of HMF. The addition of RPC to the cookies showed the highest increase of HMF (three times and six times higher than the control samples in the cookies containing 6.5 % and 12.9 % RPC) in our test series. These results were not surprising since the presence of precursors as glucose can strongly enhance the formation of HMF. Furthermore, the combination of amino groups, fiber, and also additional reactive intermediates from RPC, led to even higher levels of HMF in the cookies containing RPC than in the samples with the other two ingredients. For the cookies containing the alkaline extract, similarly to the addition of RPC a physicochemical influence on HMF formation was envisaged (**Figure 1**). Firstly, the addition of reactive intermediates as glucose or 3-deoxyglucosone deriving from the extract can contribute to the final concentration of HMF in the alkaline extract. Secondly, the presence of soluble proteins in the extract reduced the water activity in the cookies (Gallagher, Kenny & Arendt, 2005). By decreasing the water activity down to 0.75, the relative reaction rates of the enzymatic browning is increased and this property had a promoting impact on HMF formation in the cookies containing the alkaline extract thus leading HMF levels up to 2.5 times higher.

HMF levels increased by adding different concentrations of the fiber extract derived from RPC to the cookies leading to a maximum increase of HMF by adding 5.2 % fiber extract to the cookies (**Figure 1**). The more fiber extract was added the higher the HMF levels found in the model cookies, showing a linear correlation between fiber extract concentration and HMF levels. The formation of HMF in the cookies containing fiber extract were ascribed to the fiber WHC following a similar mechanisms for the pyrolysis and dehydration of precursors, as 3-deoxyglucosone, 3,4-dideoxyosone and fructofuranosyl cation (Perez Locas & Yaylayan, 2008).

Concerning the results on acrylamide, different effects according to the recipes were observed. Acrylamide increased up to 45.3% and 66.9% in the cookies containing 6.5 % and 12.9 % RPC, respectively, as reported in **Figure 2**. The addition of precursors played a key role as observed for HMF formation. On one hand, glucose in RPC favored the formation of the first intermediate the so-called Schiff base (in equilibrium with *N*-glycosylasparagine), upon the Amadori rearrangement, the *N*-(1-deoxy-D-fructos-1-yl)-L-asparagine was formed that through a decarboxylation step led to the increase in acrylamide concentration (Yaylayan, Wnorowski & Perez Locas, 2003). On the other hand, cold pressed RPC contains approximately 28.0 % crude protein, 11.2 % crude fiber, and 17.8 % crude fat (monounsaturated fatty acids = 58.5−68.0%, polyunsaturated fatty acids = 24.7−33.9%) that could actively contribute to the final concentration of acrylamide (Leming & Lember, 2005). Previous studies indicated that the fatty acid composition of heated food affected acrylamide levels by the reaction of lipid oxidation products with asparagine, thus forming acrylamide (Arribas-Lorenzo, Fogliano & Morales, 2009; Capuano, Oliviero, Açar, Gökmen & Fogliano, 2010). As RPC is rich in monounsaturated fatty acids and polyunsaturated fatty acids it is likely that more acrylamide is formed in the cookies containing RPC due to the formation of acrylamide via two pathways: the Strecker degradation of *N*-(1-deoxy-D-fructos-1-yl)-L-asparagine and the reaction of asparagine with lipid oxidation products from fatty acids.

Acrylamide levels in the cookies containing the fiber extract were controversial, showing an increment of acrylamide up to 38% at the lowest fiber extract concentration and slightly lower acrylamide levels at higher fiber concentrations (4.1% and 4.4% in cookies containing 2.6 % and 5.2 % fiber extract, respectively).

The addition of alkaline soluble protein extract from RPC significantly reduced the acrylamide content, down to 24.2% and 39.6% in the cookies containing 6.5 % and 12.9 % of the extract, respectively as highlighted in **Figure 2**. The reduction of acrylamide in our model cookies containing the alkaline extract can be ascribed to three possible effects: (a) the phenolic ring post-oxidative reaction with amino acids as reported in **Figure 3** (Guerra & Yaylayan, 2014); (b) the elimination of acrylamide via Michael addition with free amino acids containing a nucleophilic side chain, as outlined in **Figure 4** (Adams, Hamdani, Lancker, Méjri & De Kimpe, 2010; Bråthen, Kita, Knutsen & Wicklund, 2005) and (c) the presence of polypeptide chains, whose thiol or amino groups can act as nucleophiles, thus eliminating acrylamide. Antioxidant substances can influence the formation of MRPs (Troise, Fiore, Colantuono, Kokkinidou, Peterson & Fogliano, 2014); in particular, phenolic rings with an *o*-dihydroxy function can hinder the formation of MRPs in two ways. Firstly, methylglyoxal and glyoxal can be trapped in an aromatic substitution reaction by the phenolic ring thus controlling the formation of reactive carbonyl species, as acetol or methylglyoxal, glyoxal, as well as 3-deoxyglucosone, the key precursor of HMF formation (Totlani & Peterson, 2007). Secondly, quinones can be formed in the presence of oxidants such as iron and ascorbic acid which are able to react with the side chain of nucleophilic amino acids (Guerra & Yaylayan, 2014). Rapeseed is rich in polyphenols such as isoflavanoids and cinnamic acid derivatives that in presence of pro-oxidizing agent can be converted into quinone then react with free amino groups (Naczk, Amarowicz, Sullivan & Shahidi, 1998). Anyway, whether polyphenols derived from rapeseed significantly affect the formation of MRPs in a cookie model system should be further investigated in order to depict the proper balance between promoting effects due to the

sequestration of water or to the addition of reactants and precursors and the possible reducing effects related to the polyphenols bound to the fiber or to the addition of free nucleophiles. In this respect, the cooking of the biscuits can promote the cleavage of fiber bound polyphenols and promote their oxidation into quinone.

3.3 Acrylamide elimination and Michael adducts formation

The fate of acrylamide was evaluated also toward the possible routes of its elimination, in particular via Michael addition mediated by free amino acids. The presence of acryloyl group is the prerequisite for the Michael addition hence to the formation of typical acrylamide-nucleophiles adducts. The presence of free amino acids with their amino group in position α as in the case of glycine, the two amino groups in position α and ε of lysine, cysteine with a thiol group, the pyrrolidine group of proline and the indole group of tryptophan were investigated as possible candidates for the Michael reaction and tentatively identified by high resolution mass spectrometry (Stadler et al., 2004). In Supplementary **Table 2,** the five adducts, namely Cys-acrylamide, Pro-acrylamide, Lys-acrylamide, Gly-acrylamide and Trp-acrylamide, their chemical formulas and exact masses were outlined. According to chemical mechanisms of Michael addition in **Figure 4** and to the chemical structures of the adducts; an in-house database was developed by considering free amino acids and their side chains as possible nucleophiles. The results revealed that among the five compounds only the thiol group of cysteine readily reacted with acrylamide in all the samples. In **Figure 5** the effects of alkaline soluble protein extract was reported: the addition of protein promoted the formation of the adducts via the release of free amino acids during the baking process or increasing the concentration of free amino acids in the recipe. In particular, the area counts of Cys-acrylamide was 47.1% and 105.0% higher than the control sample in presence of 6.5% and 12.9% of alkaline extract, respectively. The addition of fiber also promoted the formation of Cys-acrylamide with an increase that ranged from 35.8% and 54.4% towards the control samples revealing that the alkaline extraction was an effective tool in the formation of Cys-acrylamide

adducts, but in the case of RPC the presence of glucose determined higher concentration of both HMF and acrylamide. These results confirmed previous evidences in aqueous model systems highlighted by Koutsidis and co-workers (2009) by monitoring the adduct Cys-acrylamide and by Adams and co-workers (2010) that investigated the formation of the mono-addition product cysteine-S-b-propionamide as well as the double addition products. The chemical mechanisms beside the reaction between acrylamide and nucleophiles were reported by Stadler and co-workers: acrylamide may react with soft nucleophiles according to the hard and soft acid base (HSAB) theory, following Michael type addition reaction (Stadler et al., 2004) Along with model system, Michael addition can occur also in foods. The same mechanisms were hypothesized by Narita and co-workers in canned coffee added with cysteine (Narita & Inouye, 2014), while Capuano (2009) and co-workers demonstrated that glycine plays a key role for the reduction of acrylamide during the heating treatment of bread crisps. Moreover, in presence of free amino acids, similar mechanisms were outlined also during digestion, as recently revealed during *in vitro* multistep enzymatic digestion of thermally processed foods (Hamzalıoğlu & Gökmen, 2015). Beside the presence of free nucleophiles as possible reactants in the Michael addition, it is necessary to remark that also polypeptide chains, and in particular soluble proteins added in the three recipes can exert an active role in the Michael addition thus contributing to the elimination of highly reactive acryloyl groups.

4. Conclusions

RPC contains a variety of compounds able to affect the formation of MRPs during heat treatment of foods. It was shown that the alkaline extract from RPC used in this study enables the reduction of acrylamide in our model system down to 39.6%. This observation can be related to the elimination of acrylamide via the Michael addition with nucleophilic amino acids, in particular with the thiol group of cysteine side chain. Moreover, acrylamide precursors may react with polyphenols present in the protein extract used in this study and further investigations will be addressed in this direction

in order to highlight the potential effects of polyphenols bound to the fiber in reacting with amino group of asparagine. HMF concentration increased in the cookies containing the three ingredients from RPC, probably as result of the addition of precursors or as a consequence of technological aspects. This effect was associated to the WHC of the fiber and protein, as well as to the addition of reactants and precursors, such as glucose in the case of RPC. The purity of the fiber extract and alkaline soluble protein extract is the key point to emphasize the potential of this ingredient in controlling the formation of potentially toxic MRPs. Furthermore, we propose that including the investigation of a polyphenol-rich extract derived from RPC on the formation of acrylamide and HMF can be a promising tool to tune MR in baked foods.

The authors declare no conflicts of interest and do competing financial interest

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Tables legend

Table 1: Recipes used for the different cookies.

Supplementary Tables legend

Supplementary Table 1: Characterization of soluble protein, fiber and RPC used as ingredients.

Supplementary Table 2: High resolution mass spectrometry setup for acrylamide and amino acids adducts and their tentative chemical formula. Error (ppm) was calculated as the ratio between the difference of the theoretical mass minus the experimental mass and the theoretical mass. This ratio was multiplied per one million to obtain the ppm.

Figures legend

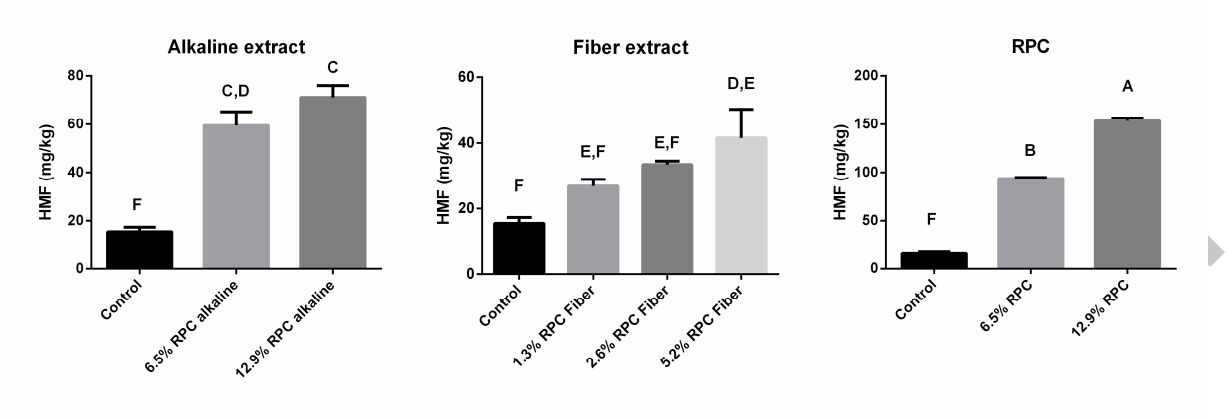
Figure 1: acrylamide concentration in the recipes in presence of 6.5 % and 12.9% of alkaline extract, 1.3%, 2.6 %, 5.2% of fiber extract and 6.5 % and 12.9% g of RPC. Significant differences were determined by ANOVA analysis and Tukey test ($p \leq 0.05$). Different letters indicate significant differences.

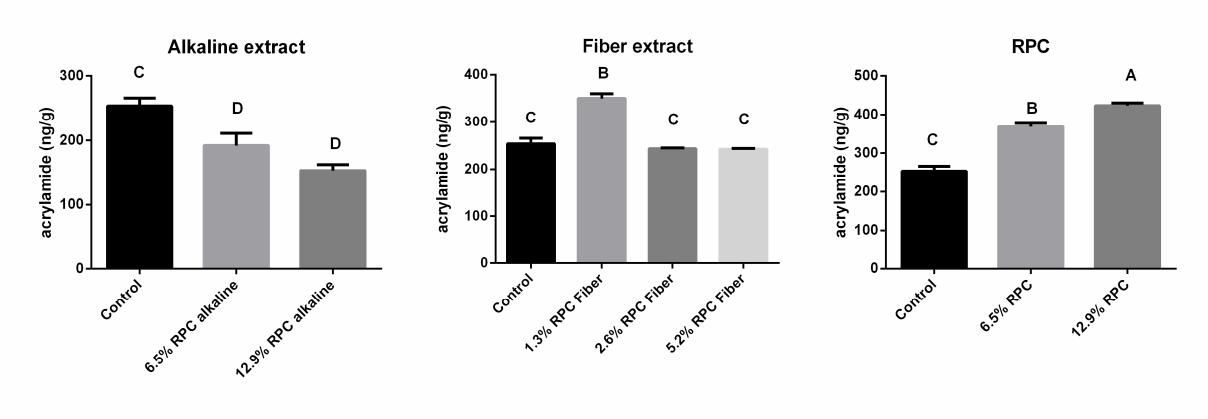
Figure 2: HMF concentration in the recipes in presence of 6.5 % and 12.9% of alkaline extract, 1.3%, 2.6 %, 5.2% of fiber extract and 6.5 % and 12.9% g of RPC. Significant differences were determined by ANOVA analysis and Tukey test ($p \leq 0.05$). Different letters indicate significant differences.

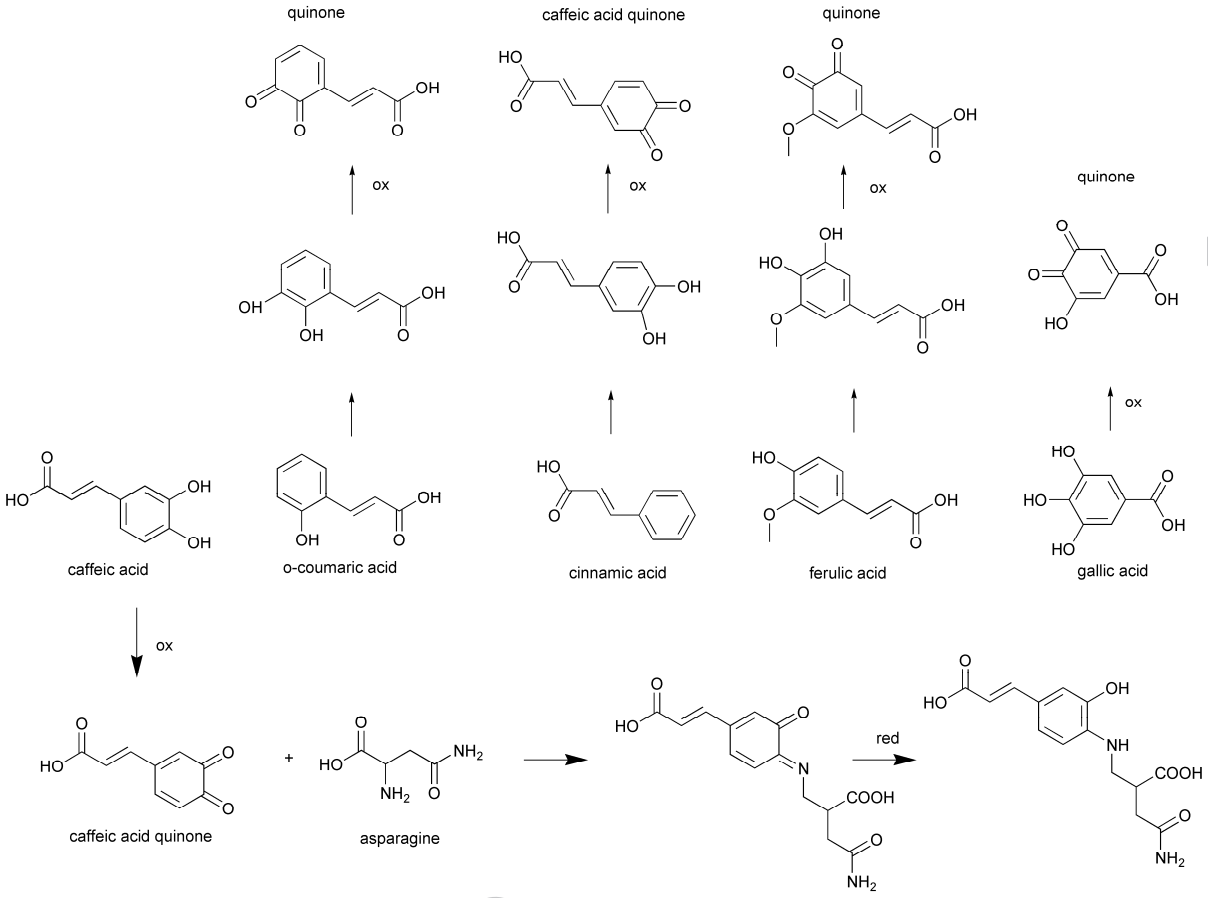
Figure 3: Hypothesized mechanisms of acrylamide reduction, post-oxidative reaction of phenolic rings with free amino group according to Guerra and Yaylayan (2014). In the center the main phenolic acid compounds present in RPC, at the top their quinone structures. At the bottom the hypothesized reaction between asparagine and caffeic acid quinone.

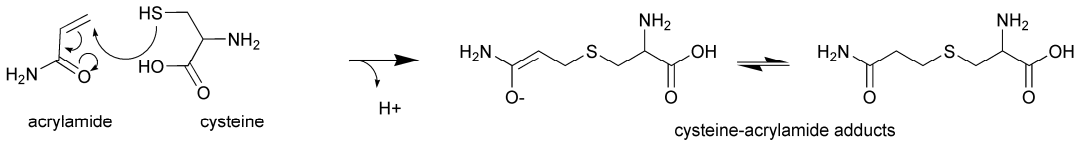
Figure 4: Mechanism of Michael addition between cysteine and acrylamide. On the right, the tentative structures of the adducts formed by glycine, proline, tryptophan, lysine.

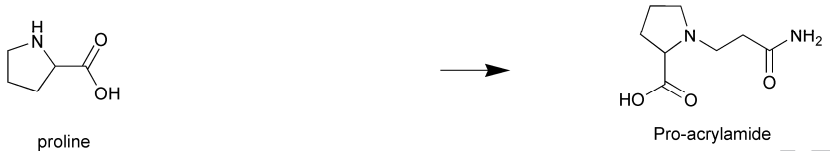
Figure 5: Area counts of the adduct Cys-acrylamide in the recipes. Significant in Cys-acrylamide adducts area counts were determined by ANOVA analysis and Tukey test ($p \leq 0.05$).

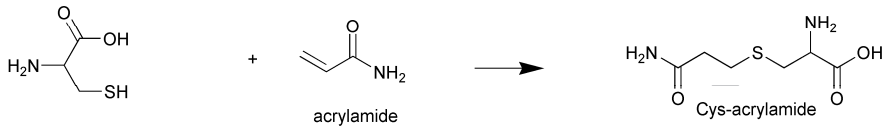


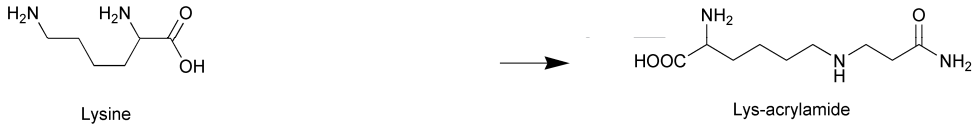


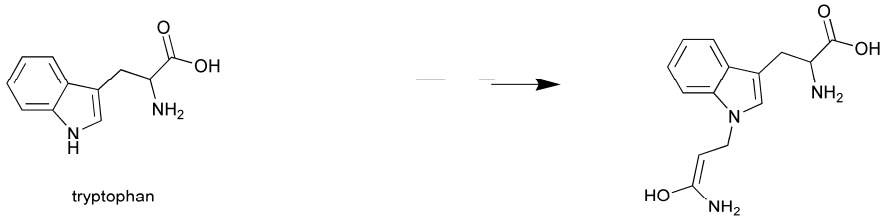












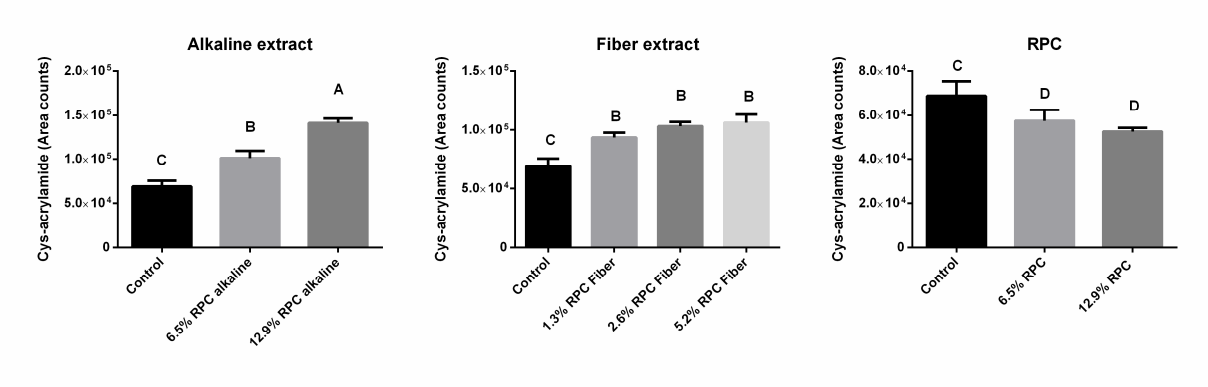
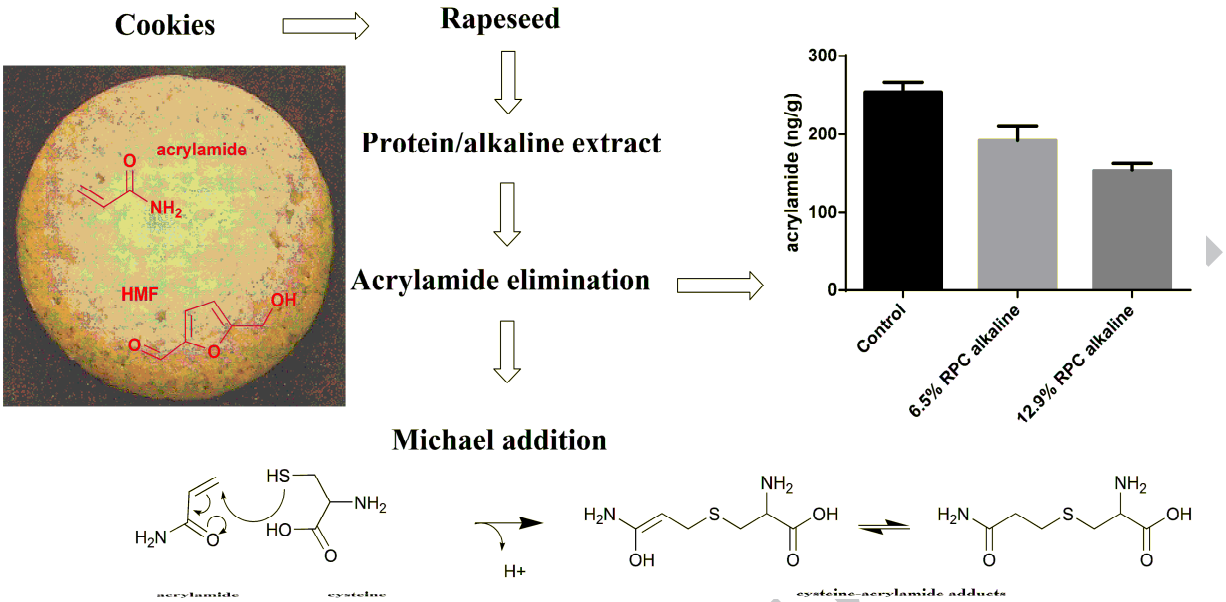


Table 1: Recipes used for the different cookies.

	Control cookies	**Alkaline extract cookies**	**Fiber extract cookies**	**RPC cookies**
Wheat flour (g)	80.0	70.0 and 60.0, respectively	78.0; 76.0, and 72.0, respectively	70.0 and 60.0, respectively
Alkaline extract (g)	/	10.0 and 20.0, respectively	/	/
Fiber extract (g)	/	/	2.0; 4.0, and 8.0, respectively	/
Native press-cake (g)	/	/	/	10.0 and 20.0, respectively
Palm oil (g)	20.0	20.0	20.0	20.0
Sucrose (g)	35.0	35.0	35.0	35.0
$NaHCO_3$ (g)	0.8	0.8	0.8	0.8
NaCl (g)	1.0	1.0	1.0	1.0
NH_4HCO_3 (g)	0.4	0.4	0.4	0.4
Water (ml)	17.6	17.6	17.6	17.6



Highlights

- Rapeseed press-cake extracts were added to cookies model systems
- Alkaline extract was able to reduce acrylamide formation in cookies.
- Michael addition controls the elimination of acrylamide in cookies.